β-1,3 GLUCAN ACTIVATION OF CRUSTACEAN HEMOCYTES *IN VITRO* AND *IN VIVO*

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ABSTRACT

The effects of β-1,3 glucans on the hemocytes of the freshwater crayfish, *Astacus astacus,* and the shore crab, *Carcinus maenas,* were studied *in vitro* and *in vivo* to determine the role of the prophenoloxidase activating system, in the cellular defense reactions of crustaceans.

In vitro, phagocytosis of the bacterium, *Moraxella* sp. was significantly raised by addition of laminarin, a β-1,3 glucan, simultaneously with the test particles to the hemocytes in monolayer cultures. Both the proportion of cells ingesting one or more bacterial particles and the number of bacteria taken up by individual cells were increased, and the responses were found to be time dependent and dose related. Glucose, dextran, cellulose, and chitin had no stimulatory influence on the cells, and the agglutination of erythrocytes by crab hemocytes or serum was unchanged by glucan incubation. Examination of the monolayers under phase contrast microscopy, revealed that the glucans induced degranulation and occasionally lysis in the crayfish hemocytes, and vacuolation in the crab hemocytes.

In vivo, injection of β-1,3 glucans (0.2 mg laminaran/ml hemolymph) into the hemocoel of *A. astacus* or *C. maenas* caused a rapid, marked reduction in the number of circulating hemocytes, indicating that a cellular defense reaction was initiated. Since prophenoloxidase in the hemocytes is specifically activated by β-1,3 glucans, and in the activated form phenoloxidase is "sticky," it is suggested that certain proteins of the prophenoloxidase activating system may serve as opsonins, and possibly constitute an important recognition mechanism in crustaceans.

INTRODUCTION

Lacking immunoglobulins, invertebrates are potentially useful models for analyses of the non-specific immune processes, such as Ig-independent phagocytosis and cytotoxicity. But, despite the numerous observations of the various humoral and cellular defense reactions in different species (Ratcliffe and Rowley, 1979), the molecular basis for the recognition of non-self materials is still not fully understood. Recently, however, β-1,3 glucans, carbohydrates from fungal cell walls which have a potent stimulatory action on the reticuloendothelial system of mammals (Kokoshis *et al.,* 1978; Di Luzio, 1979), have been found to elicit melanization (Unestam and Söderhäll, 1977; Söderhäll and Unestam, 1979) and coagulation reactions in the hemolymph of arthropods (Kakinuma *et al.,* 1981; Morita *et al.,* 1981; Söderhäll, 1981). In crustaceans and insects, the glucans exert their effect by specifically activating prophenoloxidase, a proenzyme involved in melanin synthesis, in the hemocytes or plasma, through a complex enzyme cascade including at least one serine protease (Ashida, 1981; Söderhäll, 1981; Söderhäll, 1983). In arthropods, melanin

deposition frequently accompanies the host cellular responses to wounding, foreign implants, parasites or invading micro-organisms (Salt, 1970; Ratcliffe and Rowley, 1979) and, in insects (Salt, 1970; Vey, 1979), phenoloxidase appears to be associated with host resistance. Experiments were therefore carried out to determine the effect of β-1,3 glucans on the hemocytes and to examine the role of prophenoloxidase in hemocyte activity. Using the freshwater crayfish, *Astacus astacus,* for which the prophenoloxidase activating system has been defined (Söderhäll, 1982, 1983), and the shore crab, *Carcinus maenas,* as convenient hosts, phagocytosis, a phenomenon universal throughout the animal kingdom was studied *in vitro.* The influence of the glucans on the hemocytes *in vivo* and the agglutination of test particles *in vitro* were also investigated since in invertebrates agglutinins are thought to function as recognition factors or opsonins (McKay and Jenkin, 1970; Renwrantz and Cheng, 1977).

<div align="center">MATERIALS AND METHODS</div>

Animals

Specimens of *Astacus astacus* were collected from Lake Vallsjön, Småland, Sweden, and kept as described in Söderhäll and Unestam (1979) *Carcinus maenas* were obtained, with creels, from Balloch Bay, Firth of Clyde, Scotland and housed as previously reported (Smith and Ratcliffe, 1978). Only healthy, intermoult animals were used for experimental purposes.

Culture media

Balanced salt solutions, made up to resemble the ionic composition of *A. astacus* or *C. maenas* hemolymphs were used for the culture of the hemocytes *in vitro* and other experiments. The composition of crayfish saline (CFS) was: 0.2 mM NaCl; 5.4 mM KCl; 10 mM CaCl$_2$; 2.6 mM MgCl$_2$ and 2.0 M NaHCO$_3$, pH 6.8; and the composition of *Carcinus* saline (CS) was as given in Smith and Ratcliffe (1978). Preliminary tests confirmed that hemocyte viability, determined by trypan blue exclusion, remained as high as 98% for 6 h at 20°C in these salines.

Glycans

Laminarin (Calbiochem), a β-1,3 glucan, prepared as described in Söderhäll and Unestam (1979), was suspended in sterile CFS or CS at an initial concentration of 1%, dissolved by heating and then diluted to the required strength in CFS and CS. Glucose (Merck), dextran (T40, Pharmacia Uppsala), cellulose (Avicel, OP 300, Kebo, Stockholm) and chitin (prepared as described in Söderhäll and Unestam (1979) were similarly dissolved but to concentrations of 0.2%. In addition, a crude β-1,3 glucan fraction (Zs) was also obtained from the supernatant of a 1% zymosan (Sigma Chemical Co., Kingston-upon-Thames, Surrey) suspension (Söderhäll and Unestam, 1979) in double distilled water, and was used in the prophenoloxidase estimation assays for *C. maenas.*

Bacteria

The gram negative bacterium, Moraxella sp. (NCMB 308) was grown and prepared as described in Smith and Ratcliffe (1978). The washed bacteria were suspended in CFS or CS at a concentration of 2.0×10^7 ml^{-1}, before dilution with an equal volume of either the desired glycan solution or the appropriate culture saline.

Preparation and treatment of monolayers

Crayfish hemocyte monolayers were prepared by mixing *ca.* 200 μl of hemo-lymph, bled from the abdominal hemocoel with an 18 g needle, to 100 μl of CFS, containing cysteine (Sigma) (15 mg ml^{-1}) as an anticoagulant, on clean pyrogen-free coverslips. The coverslips, in sterile, plastic multiwell trays, were left to stand at 20°C for 15 min to allow the cells to settle and attach to the glass surfaces, before being washed with two 2.5 ml volumes of sterile, filtered CFS. Similar monolayers were prepared for *C. maenas* according to the method described by Smith and Ratcliffe (1978).

Initially, to observe the effects of β-1,3 glucans on crayfish and crab hemocytes *in vitro*, freshly prepared monolayers were overlaid with 100 μl of 0.1% laminarin or, in the case of controls, with 100 μl of CFS or CS. Following incubation at 20°C for 1 h, the hemocytes were fixed in 2.5% glutaraldehyde (Agar Aids, Bishop Stort-ford, Herts) in CFS or CS (pH 6.8 or 7.4) and examined under phase contrast optics of a Leitz Dialux 20 microscope.

Next, to examine the influence of glycans on the phagocytic capacity of *A. astacus* and *C. maenas* hemocytes *in vitro*, two series of monolayers were prepared for each animal. One series was given 100 μl doses of *Moraxella* sp. in 0.1% lam-inarin, while the second received 100 μl of *Moraxella* in CFS of CS. For comparison, additional monolayers were inoculated with 100 μl of bacteria suspended in 0.1% glucose or the appropriate culture saline, and, to provide some information as to the specificity of the cellular responses to the glycans, hemocyte monolayers, pre-pared from three crayfish, were challenged with 100 μl of bacteria in 0.1% dextran, cellulose or chitin, and, for the controls, crayfish saline.

After inoculation, the coverslips were placed in a moist chamber and incubated on a rocking platform for 2 h at 20°C. The cultures were then removed, rinsed twice with CFS or CS and fixed in 2.5% glutaraldehyde (15 min) before examination under phase contrast optics.

In a separate experiment, designed to evaluate the dose response, bacterial sus-pensions made up in 0.01% or 0.001% laminarin were added, in 100 μl doses, to preformed crayfish or crab monolayers, and the cultures incubated, rinsed, fixed and examined as above. Control monolayers, prepared from the same animals, were similarly treated but with bacterial suspensions made up in CFS or CS only.

Finally, to investigate the kinetics of the cellular reaction, hemocyte cultures of *A. astacus* or *C. maenas* were preincubated with laminarin (100 μl of a 0.1% so-lution) or, in the controls, with saline, for 1 h at 20°C prior to addition of 100 μl of the bacterial suspension. The monolayers were incubated, rinsed and fixed as described above.

Quantification of phagocytosis and analysis of results

Phagocytosis of bacteria in the experimental, *i.e.* glycan treated, and control, saline treated, monolayers was quantified by scoring the number of cells containing one or more intracellular particles (the percentage phagocytosis), and by recording the number of bacteria ingested per 100 hemocytes. Intracellular bacteria were dis-tinguished from extracellular, adherent forms using the criteria described by Smith and Ratcliffe (1978). A minimum of 400 cells was observed on randomly chosen areas from each of three coverslips for each treatment and every animal, and, to eliminate the bias inherent in this method of assessment, all counts were made blind. Unless otherwise stated, groups of 5–10 crayfish or crabs were used in each experiment.

From the triplicate coverslips, the median values for the percentage phagocytosis and the number of ingested particles were recorded for the experimental and control treatment of every animal. Differences in the rate of uptake between glycan and saline incubated monolayers were analysed statistically with the Walsh test for related samples (two tailed), and differences in the rates of uptake between glucan concentrations were analysed with the Mann Whitney U test (one tailed) (Siegel, 1956). The specified level of significance for both tests was ≤0.05.

To emphasize the change in the cellular responses to the bacteria induced by glycan treatment, the results were also expressed as the enhancement index (EI). This was derived for each animal from the formula:

$$\frac{\text{Rate of uptake in experimental (glycan treated) cultures}}{\text{Rate of uptake in control (saline treated) cultures}} \times 100$$

Enhanced uptake is thus shown by EI values greater than 100, whereas suppression of phagocytosis is indicated by values for the EI below 100.

Preparation of crab hemocyte lysate supernatant (HLS) and assay of phenoloxidase activity

Although in crayfish β-1,3 glucans are known to activate prophenoloxidase within the hemocytes (Söderhäll and Unestam, 1979; Söderhäll, 1981), equivalent events have not been demonstrated for *C. maenas*. In this investigation, therefore, hemocyte lysates were prepared from crab hemolymph, activated with β-1,3 glucans and then assayed for phenoloxidase and protease activity.

For the HLS, hemocytes were harvested from six crabs by withdrawing 2.25 ml of hemolymph from each crab, into a 5 ml syringe containing 2.5 ml of ice-cold cacodylate buffer (10 mM sodium cacodylate; 5 mM CaCl$_2$; 0.25 M sucrose and 0.1 M sodium citrate), pH 7.0. The samples were pooled, centrifuged at 800 g for 10 min and the pellet washed once with 50 ml of citrate-depleted cacodylate buffer, pH 7.0, at 4°C. The hemocyte pellet was then homogenized in 4.0 ml of cacodylate: CaCl$_2$ buffer (10 mM sodium cacodylate; 5 mM CaCl$_2$) pH 7.0, and the supernatant remaining after centrifugation at 40,000 g for 30 min (4°C) was used as the enzyme source. For the hemagglutination assays (see below), the hemocyte pellet was homogenized in 4.0 ml of CS at 4°C before centrifugation at 40,000 × g (10 min) and storage at −4°C.

Phenoloxidase activity in glucan or saline incubated HLS was determined using L-dihydroxyphenylalanine (L-dopa) as substrate (Söderhäll, 1981). In the experimental tubes 400 μl of crab HLS was preactivated with 200 μl of 0.1% laminarin or Zs, and 200 μl of 200 mM MgCl$_2$ for 1 h at 20°C; 200 μl of this reaction mixture was then added to 200 μl of L-dopa (4 g l^{-1}) with 400 μl of cacodylate buffer (10 mM sodium cacodylate; 5 mM CaCl$_2$) pH 7.0, and after 5 min at 20°C, the absorbance was read at 480 nm. Control mixtures, in which distilled water or CS was substituted for the glucans in the activation step, were run parallel to the experimentals, and for all samples, phenoloxidase activity was expressed in units: one unit representing the amount of enzyme required to produce an increase in absorbance of 0.001 min^{-1}. Preliminary tests confirmed that activity was proportional to enzyme concentration and linear with respect to time.

Assay of HLS protease activity

Protease activity was assayed using the synthetic chromogenic peptide, Bz-Ile-Glu-(γ-O-Piperidyl)-Gly-Arg-PNA-HCl (AB Kabi Peptide Research, Mölndal,

Sweden), as substrate (Söderhäll, 1983). HLS was first activated with laminarin or Zs as above, and 200 μl of the reaction mixure was incubated with μl of 1.5 mM chromogenic peptide for 30 min at 20°C. The reaction was terminated by addition of 100 μl of 50% acetic acid, and the released p-nitroaniline measured spectrophotometrically at 405 nm. Enzyme activity was expressed as the rate of change in absorbance at this wavelength.

Protein determination

The protein content of crab HLS was determined according to the method of Lowry *et al.* (1951) using bovine serum albumin for the standards.

All biochemical measurements were repeated at least three times.

Titration of hemagglutinins

Alsevers stored sheep or horse erythrocytes (Gibco Biocult, Renfrew, Scotland) and fresh, citrated mouse or human type A blood samples were used to examine the effect of β-1,3 glucans on the agglutinating property of crustacean serum or HLS. Before use, the erythrocytes were washed three times in phosphate buffered saline pH 6.8 (Oxoid Ltd., Basingstoke, Hants) and resuspended in culture saline at a concentration of 4% v/v.

Crab serum was prepared from 6–8 animals as described in Smith and Ratcliffe (1978), and *C. maenas* HLS was made, in CS, as above. Aliquots of 50 μl of serum or HLS were serially diluted twofold in CS in microtitre trays (Sterilin Ltd), and to one series of wells was added 25 μl of 0.2% laminarin while a second received 25 μl of CS. For the controls, 25 μl of 0.2% laminarin or CS was mixed with 25 μl of crab saline, and finally, 25 μl of the appropriate washed erythrocyte suspension was pipetted into each well. The trays were incubated for 2 h at 20°C and the titres were recorded as the last serum or HLS dilution showing visible agglutination. Comparable measurements were not made on crayfish serum or HLS as the results of these experiments for *C. maenas* failed to reveal any changes in HA activity with glucan treatment (see Results below).

Effect of β-1,3 glucans on A. astacus and C. maenas hemocytes in vivo

As further evidence of the activating effect of β-1,3 glucans on crayfish and crab hemocytes observed *in vitro* (see Results below), preliminary *in vivo* investigations were also carried out. For these, 0.1% laminarin in CFS or CS was injected, using 28 g needles, into the unsclerotized membrane at the base of the fourth pereiopod of crayfish (measuring 70–90 mm in length) or crabs (measuring 68–72 mm across the carapace).

To compensate for the difference in the hemolymph volumes between *A. astacus* and *C. maenas,* the size of the inoculum was adjusted so that each animal received 0.2 mg laminarin per milliliter of hemolymph. Control animals were similarly inoculated but with CFS or CS only.

The crayfish were incubated in clean, aerated tap water at 13°C, and the crabs in filtered, circulating sea water at 15°C, for 30 min, 3 h or 24 h. At each time interval, groups of 5–8 animals were removed and a known volume of hemolymph withdrawn from the abdominal hemocoel, as above, into an equal volume of 2.5% glutaraldehyde. The numbers of hemocytes present in the hemolymph samples were ascertained using improved Neubauer hemocytometers, and the counts were expressed as the total hemocyte number (THC) per milliliter of hemolymph. For the
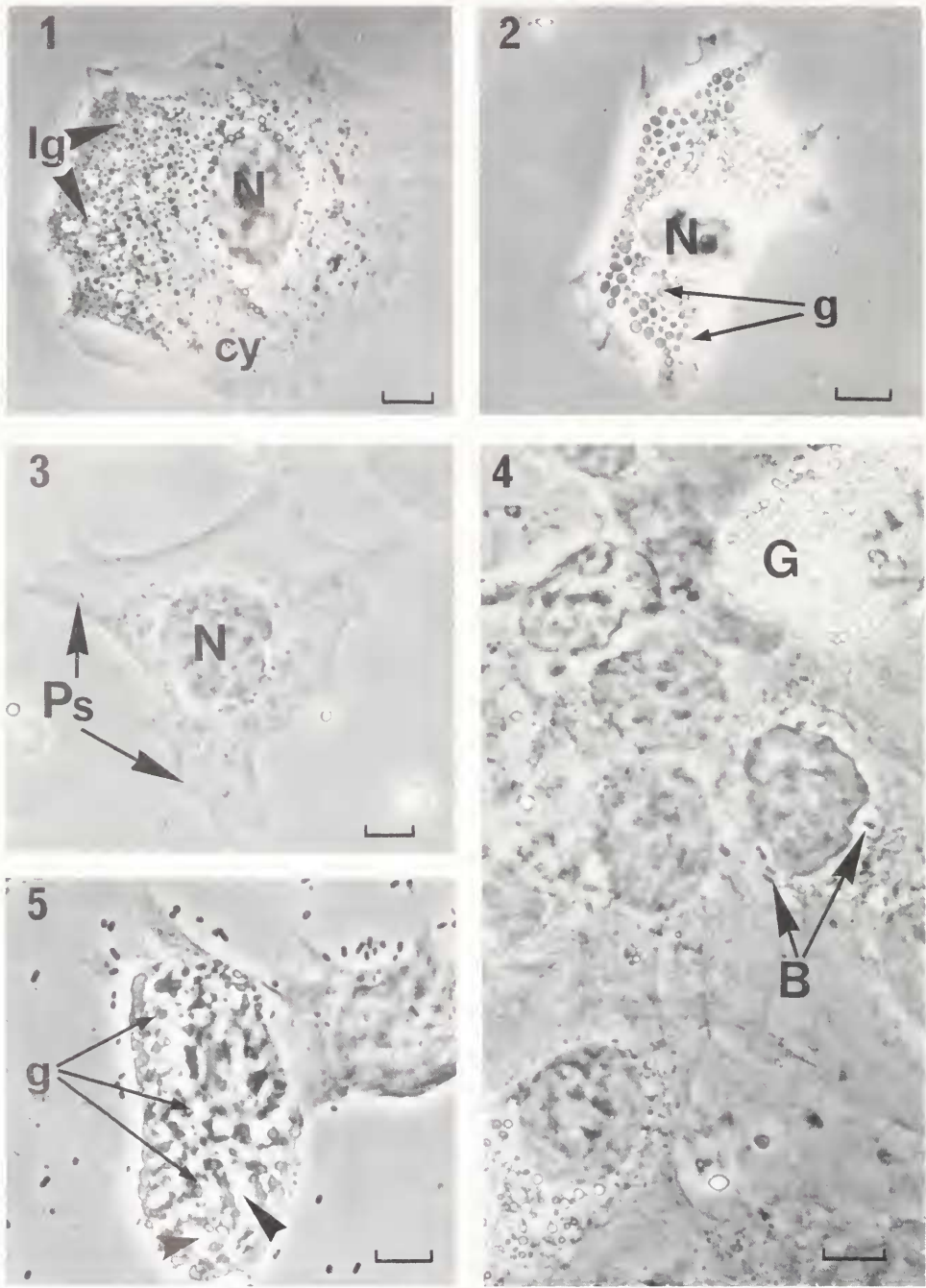
FIGURES 1-3. Hemocytes of *A. astacus* incubated for 1 h *in vitro* with CFS. Phase contrast optics.
FIGURE 1. Well spread, intact, semi-granular cell. Note the central nucleus (N) and numerous, small intracellular inclusions (Ig) in the cytoplasm (cy). Scale bar 10 μm.
FIGURE 2. Granular hemocyte containing many large, highly refractile granules (g). Nucleus (N). Scale bar 10 μm.

time zero values, similar hemolymph samples were taken from groups of untreated crayfish or crabs and the THC determined as above.

Differences in the THC between untreated, saline injected and glucan injected crayfish or crabs were analysed statistically using the Mann-Whitney U test (one tailed) (Siegel, 1956). The specified level of significance was $P \leq 0.01$.

RESULTS

Effects of β-1,3 glucans on the hemocytes of A. astacus and C. maenas in vitro

Monolayers of *A. astacus* were seen to contain three morphologically distinct hemocyte types (Figs. 1, 2, 3). The most abundant were the semi-granular hemocytes (Fig. 1), which comprised *ca.* 50% of the cells on the coverslips, and were usually flattened and well spread, with a central nucleus and a variable number of small (*ca.* 1 μm diameter) intracellular inclusions. The second cell type, the granular cells, was similar to the semi-granular hemocytes in size and shape, but always enclosed numerous, large (*ca.* 1–3 μm diameter), highly refractive granules (Fig. 2), and represented *ca.* 30% of the cells in the cultures. The remaining cells in the monolayers were the hyaline amoebocytes (Fig. 3) and these cells were distinguished from the others by the lack of large distinct cytoplasmic inclusions and the formation of long pseudopodial extensions during attachment to the coverslip surface. The monolayers of *C. maenas* were composed of flattened, well spread hyaline cells, as described previously (Smith and Ratcliffe, 1978), and incubation of crayfish or crab cultures in saline for 1 h produced no visible effects on the hemocytes (Figs. 1–4, and 6).

However, in *A. astacus,* treatment with 0.1% laminarin caused marked degranulation with consequent vacuolation in some (*ca.* 70%) of the semi-granular and granular cells (Figs. 5, 7), and occasionally, in the phagocytosis assays, prolonged exposure to the glucans resulted in lysis of *ca.* 10–20% of the cells (Fig. 8).

Granule discharge could not be observed in *C. maenas* hemocytes following glucan treatment since the granular (refractile) cells of this animal are rarely present on the monolayers (Smith and Ratcliffe, 1978). However, addition of 100 μl of L-dopa to the glucan treated monolayers resulted in dopachrome formation, showing that phenoloxidase was present on the coverslips. The hyaline (phagocytic) cells (Smith and Ratcliffe, 1978) of crabs exhibited some vacuolation in response to the glucans (Fig. 9), but the effects were usually less dramatic than in *A. astacus.*

During the phagocytosis studies, neither crayfish nor crab hemocytes showed any morphological changes to 0.1% glucose, and in crayfish, the appearance of the cells in the dextran, cellulose or chitin incubated cultures was similar to those in the saline incubated controls.

Effect of β-1,3 glucans on in vitro phagocytosis of bacteria

In vitro, phagocytosis of *Moraxella* sp. by the hemocytes of *A. astacus* and *C. maenas* was enhanced by β-1,3 glucans (Tables I, II). Addition of 0.1% laminarin

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FIGURE 3. Hyaline hemocyte central nucleus (N) and pseudopodial extensions (Ps). Note the absence of large, distinct cytoplasmic inclusions. Scale bar 10 μm.

FIGURE 4. Hemocyte monolayer of *A. astacus* incubated for 2 h with *Moraxella* sp. Most of the cells are of the hyaline type, one of which contains four intracellular bacteria (B). A granular hemocyte (G) is present but none of the cells show signs of damage, vacuolation or lysis. Phase contrast optics. Scale bar 10 μm.

FIGURE 5. Granular hemocyte of *A. astacus* incubated for 1 h in 0.1% laminarin. The cell has partially degranulated, leaving empty spaces in the hemocyte cytoplasm (arrows). Remaining granules (g). Phase contrast. Scale bar 10 μm.
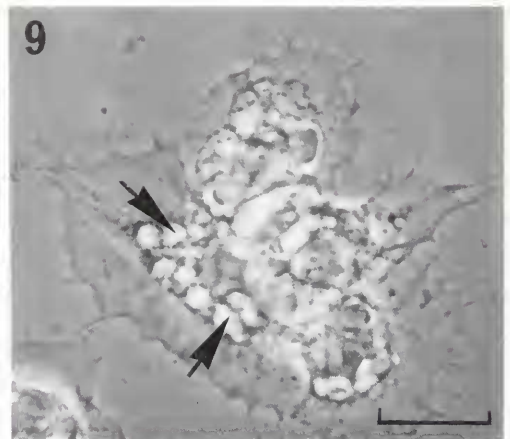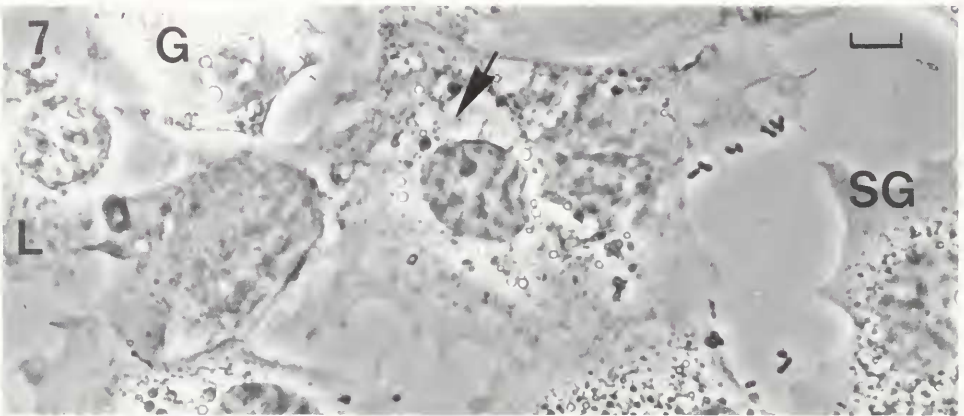
FIGURE 6. Hemocyte monolayer of *C. maenas* incubated for 1 h in CS. All the cells shown are well spread hyaline (phagocytic) types, with no sign of vacuolation or lysis. Phase contrast optics. Scale bar 10 μm.

FIGURE 7. Hemocyte monolayer of *A. astacus*, incubated for 1 h in 0.1% laminarin. Note the vacuoles (arrow) in the central cell, and the remnants of a lysed cell (L). Intact granular (G) and semigranular (SG) cells are also present. Phase contrast. Scale bar 10 μm.

TABLE I

In vitro *phagocytosis of* Moraxella *sp. by the hemocytes of* A. astacus

Treatment of monolayers	Percentage phagocytosis	EI[a]	Number of bacteria/ 100 hemocytes	EI
0.1% laminarin control[c] n = 8[d]	18.0[b] ± 4.8 } 5.6 ± 1.1 } P = 0.008	328	358 ± 60 } 223 ± 53 } P = 0.008	160
0.01% laminarin control n = 5	12.1 ± 4.3 } 3.7 ± 1.2 } P = 0.008	315	446 ± 91 } 246 ± 68 } P = 0.008	181
0.1% glucose control n = 6	5.0 ± 2.2 } 4.5 ± 1.5 } P > 0.097	111	267 ± 79 } 229 ± 56 } P > 0.097	116
0.1% dextran control n = 3	5.6 ± 3.4 } 5.3 ± 2.2 } P > 0.05	101	226 ± 97 } 201 ± 27 } P > 0.05	112
0.1% cellulose control n = 3	8.7 ± 2.4 } 5.3 ± 2.2 } P > 0.05	165	230 ± 89 } 201 ± 27 } P > 0.05	114
0.1% chitin control n = 3	5.2 ± 4.1 } 5.3 ± 2.2 } P > 0.094	85	207 ± 71 } 201 ± 27 } P > 0.094	97
preincubation[e] in 0.1% laminarin control n = 6	2.3 ± 0.6 } 2.6 ± 1.3 } P > 0.094	85	222 ± 72 } 235 ± 52 } P > 0.094	94

[a] EI as given in Materials and Methods.
[b] Values given are means ± standard deviation.
[c] Controls were incubated in CFS.
[d] n = number of animals used in experiment.
[e] Preincubation for 1 h at 20°C before challenge with bacteria in CFS.

simultaneously with the bacteria raised the percentage phagocytosis in *A. astacus* from 5.6% to 18.5% (EI = 328) and the number of particles taken up from 223 to 358 (EI = 160) (Table 1). Similar results were obtained for *C. maenas* where the EI for the percentage phagocytosis was 303 and the number of bacteria/100 hemocytes was 200 (Table II) (Fig. 10). However, in both *A. astacus* and *C. maenas,* the response was achieved only in freshly prepared cultures overlaid immediately with glucan:bacteria mixtures, and not in those left for longer than 15 min before in- oculation (data not presented). Further analyses also revealed that the response was independent of the cell density on the coverslips and the concentration of cysteine used in the monolayer preparatory stage (data not shown).

    In contrast to the glucans, glucose (0.1%) was ineffective in promoting uptake, and in *A. astacus* the percentage phagocytosis remained at 5.0% (EI = 111), with

FIGURE 8. Lysed hyaline hemocytes of *A. astacus* after 1 h incubation in 0.1% laminarin. Note the fragmented cytoplasm (cy). Nucleus (N). Phase contrast. Scale bar 10 μm.
    FIGURE 9. Hyaline hemocytes of *C. maenas,* after 1 h incubation in 0.1% laminarin. There is some slight vacuolation in the cytoplasm in the perinuclear region (arrows). Phase contrast. Scale bar 10 μm.
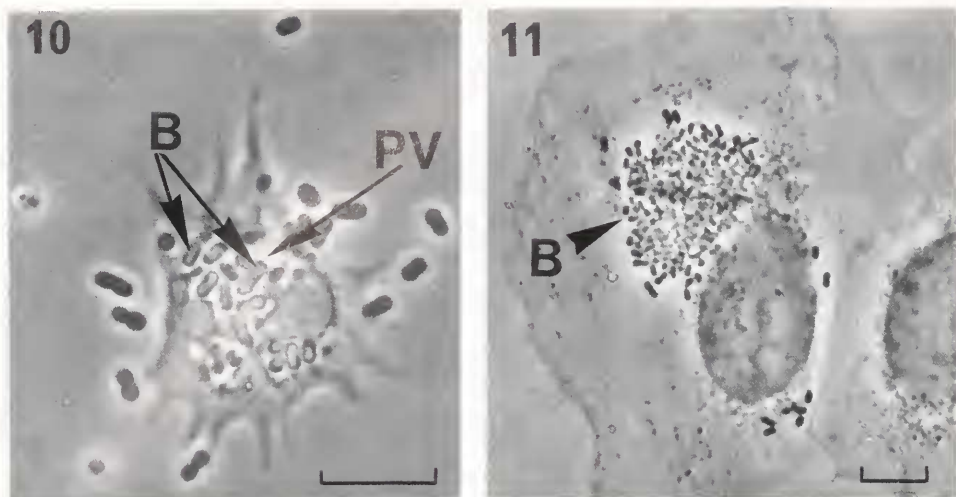
FIGURE 10. Hyaline hemocyte of *C. maenas* incubated with *Moraxella* sp. for 2 h in the presence of 0.1% laminarin. Many intracellular bacteria (B) are enclosed within large phagocytic vacuoles (PV). Phase contrast. Scale bar 10 μm.

FIGURE 11. Hyaline hemocyte of *A. astacus* incubated with 0.1% laminarin. Note the large aggregate of adherent, extracellular *Moraxella* sp. (B) associated with the cytoplasm. Phase contrast. Scale bar 10 μm.

the number of bacteria taken up at 267 (EI = 116) (Table I). The equivalent EI values for *C. maenas* were 118 for the percentage phagocytosis and 101 for the bacteria taken up (Table II).

Dextran, cellulose and chitin also failed to stimulate uptake in *A. astacus,* and in these cultures, the percentage phagocytosis was 5.6% (EI = 101) for dextran, 8.7% (EI = 165) for cellulose and 5.2% (EI = 85) for chitin, compared to 5.3% in the saline incubated controls (Table I). The corresponding values for the number of bacteria ingested were 226 (EI = 112) for dextran, 230 (EI = 114) for cellulose, 207 (EI = 97) for chitin and 201 for the controls (Table I). Cellulose and chitin were particularly unsuitable for studies of this kind because of their insolubility in the culture saline.

Regarding the dose response, treatment of *A. astacus* monolayers with 0.01% laminarin yielded an increase in the percentage uptake of 315 from 3.7% to 12.1% which was similar to the rate of phagocytosis recorded for crayfish with 0.1% laminarin ($P = 0.42$) (Table I). The number of bacteria ingested/100 cells was also increased, from 246 to 446 (EI = 181) which again resembled the response observed with 0.1% laminarin ($P = 0.47$) (Table I). In *C. maenas,* while addition of 0.001% laminarin still raised the percentage phagocytosis significantly above the level obtained for saline incubated cultures ($P = 0.008$), the degree of enhancement was only 202, a value significantly less than that achieved with 0.1% laminarin ($P = 0.002$) (Table II). The number of bacteria taken up by the hemocytes remained at 242 (EI = 112) which did not differ significantly from the number ingested in the saline incubated controls ($P = 0.097$) (Table II).

Concomitant with the enhancement of uptake of *Moraxella* sp. in crayfish and crab hemocytes treated with laminarin, was an increase in the proportion of cells associated with aggregates of ten or more extracellular bacteria (Fig. 11). Although not quantified for every treatment, in *A. astacus* monolayers the percentage asso-

TABLE II

In vitro *phagocytosis of* Moraxella *sp. by hemocytes of* C. maenas

Treatment of monolayers	Percentage phagocytosis		EI[a]	Number of bacteria/ 100 hemocytes		EI
0.1% laminarin control[c] n = 7[d]	28.4[b] ± 15.4 10.0 ± 6.6	} P = 0.008	303	545 ± 183 279 ± 43	} P = 0.008	200
0.001% laminarin control n = 7	8.9 ± 4.5 4.4 ± 2.2	} P = 0.008	202	242 ± 83 214 ± 56	} P = 0.097	112
0.1% glucose control n = 10	6.4 ± 2.7 5.4 ± 2.0	} P > 0.125	118	220 ± 41 218 ± 74	} P > 0.125	101
preincubation[e] in 0.1% laminarin control n = 5	4.3 ± 2.1 5.3 ± 3.3	} p > 0.062	82	282 ± 91 320 ± 100	} P > 0.062	89

[a] EI as given in Materials and Methods.
[b] Values given are means ± standard deviation.
[c] Controls were incubated in CS.
[d] n = number of animals used in experiment.
[e] Preincubated for 1 h at 20°C before challenge with bacteria in CS.

ciation, as defined by Smith and Ratcliffe (1978), following treatment with 0.1% or 0.01% laminarin, was 40% against *ca.* 3% in the controls. Similar differences were seen in *C. maenas* to 0.1% laminarin, and to a lesser extent with 0.001% laminarin.

It is unlikely that the glucans, alone, were responsible for the agglutination of the test particles, since *Moraxella* sp. suspensions (2.0×10^7 ml^{-1}) incubated for 2 h at 20°C in 0.1% laminarin, glucose or dextran, in the absence of crayfish or crab hemocytes, did not shown any signs of particle aggregation.

Pre-incubation of the hemocytes in 0.1% laminarin prior to bacterial challenge slightly depressed the rate of uptake in *A. astacus* and *C. maenas* (Tables I, II), and in crayfish, the percentage phagocytosis decreased from 2.6% to 2.3%, and EI of 85, with a reduction in the number of bacteria ingested from 235 to 223, an EI of 94 (Table I). In *C. maenas,* the EI values were 82 for the percentage phagocytosis and 89 for the bacteria/100 hemocytes (Table II).

Prophenoloxidase and protease activity in C. maenas *HLS*

Table III shows that *C. maenas* contains prophenoloxidase and protease in the hemocytes. Using L-dopa a substrate, phenoloxidase activity was found to be dependent on Mg^{2+} ions, and inclusion of 50 mM $MgCl_2$ with 5 mM $CaCl_2$ in the buffers provided the optimal ionic conditions for the reaction to take place (Table III). Pre-incubation of the hemocyte lysate in laminarin (250 μg ml^{-1} final concentration) enhanced the rate of enzyme activity *ca.* fourfold, with similar, but weaker, activation achieved with zymosan (Table III).

Hydrolysing activity against the synthetic chromogenic peptide, Ba-Ile-Glu(γ-O-Piperidyl)-Gly-Arg-PNA, was also slightly improved with Mg^{2+} ions, and addition of β-1,3 glucans to the experimental mixtures enhanced the activity of the enzyme *ca.* threefold.

TABLE III

Phenoloxidase and protease activities in C. maenas *hemocytes*

	Phenoloxidase activity		Protease activity	
	Units/ml	Units/mg protein	$^{\Delta A}405/30$ min	$^{\Delta A}405/$mg protein
HLS in CS (control)	260	5000	0.03	0.6
HLS in 50 mM MgCl$_2$[a]	280	5400	0.05	0.9
HLS in CS + laminarin[b]	840	16,200	0.11	2.1
HLS in 50 mM MgCl$_2$[a] + laminarin[c]	1040	20,000	0.10	1.9
HLS in 50 mM MgCl$_2$[a] + Zs	500	9600	0.08	1.5

[a] = dissolved in distilled water.

[b] = dissolved in CS (250 μg ml^{-1} final concentration during activation).

[c] = dissolved in distilled water (250 μg ml^{-1} final concentration during activation).

Phenoloxidase and protease activity assayed as described in Materials and Methods using L-dopa or the synthetic chromogenic peptide, Bz-Ile-Glu-(γ-O-Piperidyl)-Gly-Arg-PNA as substrate.

C. maenas hemocytes, therefore, appear to possess at least two of the constituent enzymes of the prophenoloxidase activating system, previously described for *A. astacus* (Söderhäll, 1981; Söderhäll, 1982, 1983).

Hemagglutination titres

Weak hemagglutinating activity was found in *C. maenas* serum, but not HLS, for sheep (titre 16–32), horse (titre 4–8) and human A (titre 32) erythrocytes. A lytic factor (titre 8) was present in both serum and HLS for mouse RBC, but neither the hemagglutinins nor the lytic factors were changed by glucan incubation.

Effect of β-1,3 glucans on A. astacus *and* C. maenas *hemocytes in vivo*

The number of circulating hemocytes (THC) in the hemolymph of untreated crayfish was found to be 4.1×10^5 ml^{-1} (Fig. 12). Injection of saline significantly raised the THC to 10.3×10^5 ml^{-1} at 30 min ($P = 0.001$) and to 9.2×10^5 ml^{-1} at 3 h ($P = 0.001$), but by 24 h, the count had returned to 6.9×10^5 ml^{-1}, which was not significantly different from untreated animals ($P = 0.016$) (Fig. 12).

Injection of laminarin, however, significantly reduced the THC to 1.7×10^5 ml^{-1} within 30 min compared to both untreated ($P = 0.005$) and saline injected controls ($P < 0.001$) (Fig. 12). The THC remained as low as 2.8×10^5 ml^{-1} for 3 h ($P = 0.064$ compared to untreated crayfish; $P < 0.001$ compared to saline injected animals) (Fig. 12), but by 24 h had recovered to 3.4×10^5 ml^{-1}, which was not significantly different from untreated ($P = 0.38$) or saline treated *A. astacus* ($P = 0.38$) (Fig. 12).

Similarly, in *C. maenas,* injection of saline was followed by an increase in the THC from 26.5×10^6 ml^{-1}, the cell count for untreated animals, to 45.7×10^6 ml^{-1} at 30 min ($P = 0.05$). After this time, the THC gradually declined to 35.0×10^6 ml^{-1} at 3 h ($P > 0.05$) and to 24.0×10^6 ml^{-1} at 24 h ($P > 0.05$) (Fig. 13). Inoculation with laminarin produced a *ca.* threefold reduction in the THC to 9.3×10^6 ml^{-1} within 30 min ($P < 0.001$ compared to untreated crabs; $P < 0.001$ compared to saline injected controls) (Fig. 13). This decline continued for 3 h, when the THC fell to 3.4×10^6 ml^{-1} ($P < 0.001$ compared to untreated *C. maenas; P < 0.001* compared to saline injected controls), but by 24 h, the THC had recovered to 31.3
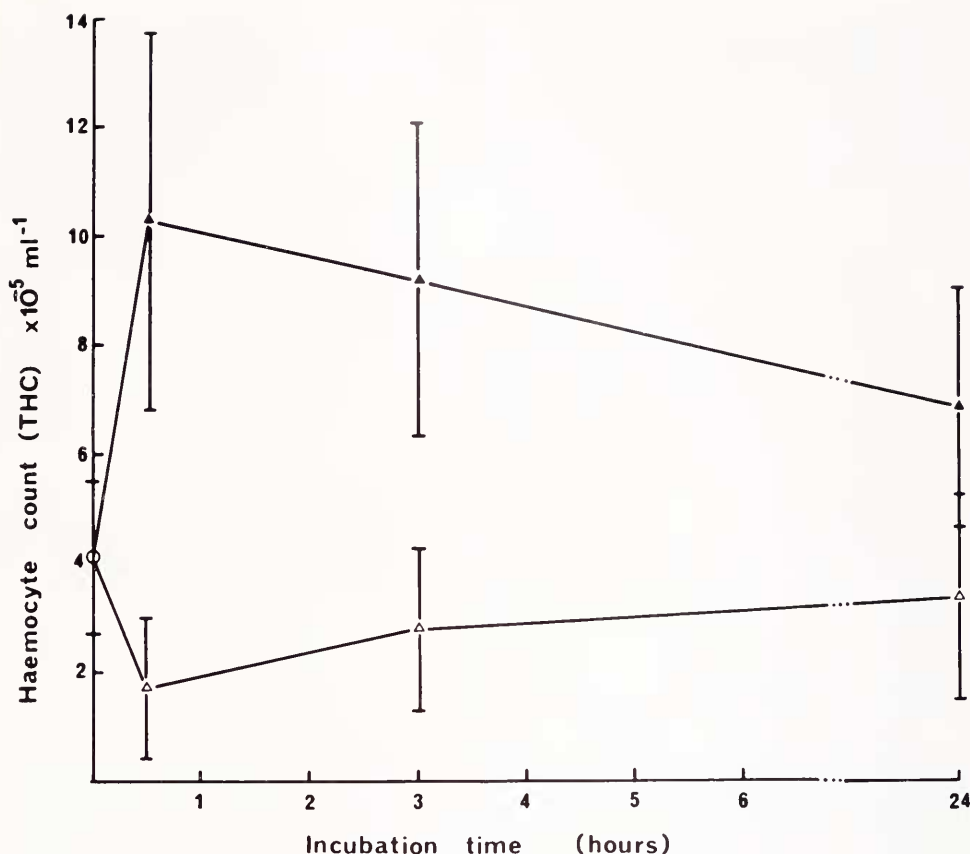
FIGURE 12. Hemocyte count (THC) of *A. astacus* injected with sterile CFS (- ▲ -) or 0.2 mg laminarin/ml hemolymph (- △ -). Injection and bleeding procedure as in Materials and Methods. At each time interval, the values given are means of groups of 8 animals ± standard deviation. The time zero count (- ○ -) was obtained from 8 untreated animals.

$\times 10^6$ ml^{-1}, which was not significantly different from untreated ($P > 0.05$) or saline injected crabs ($P = 0.03$) (Fig. 13).

Glucan injection was rarely fatal for the crayfish or crabs, but glucan inoculated animals were often lethargic, and the hemolymph samples from the experimental animals tended to clot more rapidly than those from untreated or saline injected controls.

DISCUSSION

The results presented in this paper show that β-1,3 glucans profoundly affect the behaviour of crayfish and crab hemocytes by enhancing the rate of phagocytosis of bacteria *in vitro*, and causing a marked reduction in the number of circulating cells *in vivo*. Since the responses observed *in vitro* were elicited only by laminarin, and not by glucose or other glycans, the elevated rates of uptake exhibited by the cells could not have been due simply to the nutritive properties of the β-1,3 glucans, and, as the *in vivo* reaction was similar to that seen previously in *C. maenas* following injection of bacteria (Smith and Ratcliffe, 1980a) a cellular defense reaction appears
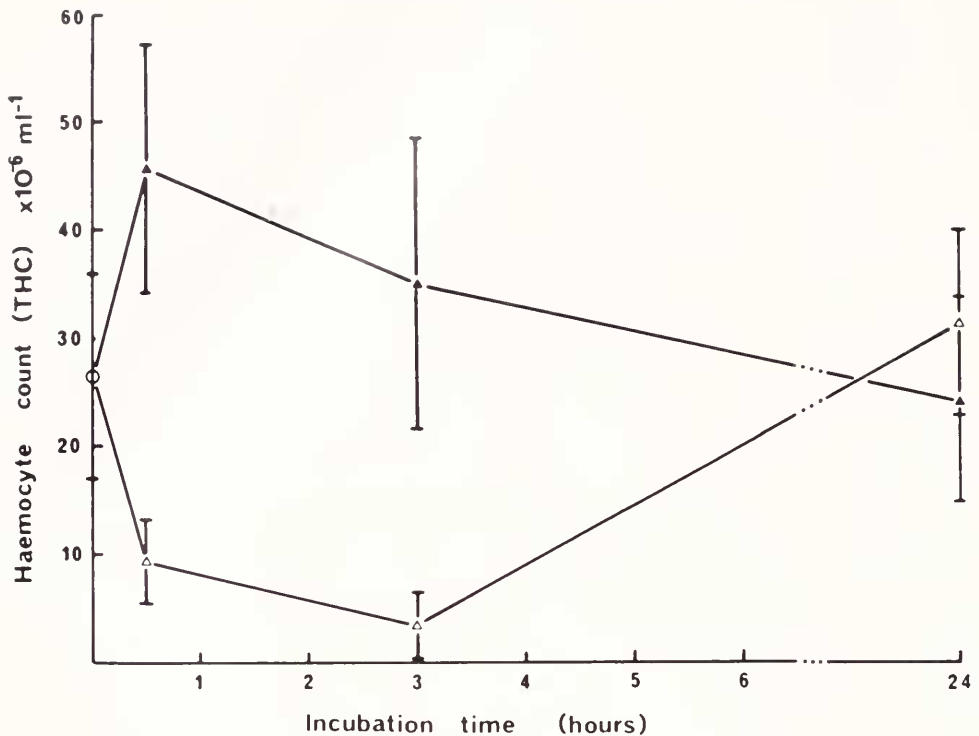
FIGURE 13. Hemocyte count (THC) of *C. maenas* injected with sterile CS (– ▲ –). Injection and bleeding procedures as in Materials and Methods, and at each time interval, the values given are means of groups of 8 animals ± standard deviation. The time zero count (– ○ –) was obtained from 10 untreated crabs.

to have been initiated. In mammals, β-1,3 glucans are known to promote a hyper-phagocytic state *in vivo* (Di Luzio, 1979) and to activate macrophages directly *in vitro* (Seljelid *et al.*, 1981), but this is the first report of their effect on cell behavior in invertebrates.

Regarding the mechanism(s) through which the glucans exert their influence in crustaceans, it is unlikely that they act through specific agglutinin mediated receptors on the hemocyte surface as proposed for opsonic phenomena reported in other vertebrates (McKay and Jenkin, 1970; Renwrantz and Cheng, 1977), since agglu-tinating activity against *Moraxella* sp. is absent from *C. maenas* serum (Smith and Ratcliffe, 1978), and, in the present study, changes in the titre of erythrocyte ag-glutinins were not detected following glucan incubation. Instead, in *A. astacus*, β-1,3 glucans are known to specifically activate prophenoloxidase, in the hemocytes, through a complex enzyme cascade involving at least one serine protease (Söderhäll, 1982, 1983) and a similar system, also activated by β-1,3 glucans, appears to exist in *C. maenas*. After activation, phenoloxidase is sticky and will attach to many different types of foreign surface including glass, plastic or fungal spores (Söderhäll *et al.*, 1979), so that it could have facilitated the binding and subsequent ingestion of the bacteria by the cells in the present study. Certainly, in *A. astacus* the glucans were seen to promote degranulation of the cells in the monolayers, and although it was impossible to observe equivalent events in *C. maenas* hemocytes, addition

of L-dopa to the crab monolayers following glucan treatment confirmed the presence of phenoloxidase on the coverslip surface. Further support for the opsonic role of the hemocyte proteins has also recently been provided by Söderhäll, Vey, and Ramstedt (submitted for publication) who showed that crayfish haemocytes mount stronger encapsulation responses *in vivo* to fungal spores coated with hemocyte lysate supernatant than to fungal spores coated in plasma or buffer. Also in *C. maenas*, electron-dense material, identified as melanin, has been noted around *Moraxella* sp. enclosed within hemocyte clumps 24 h after injection of the bacteria into the hemocoel (Smith and Ratcliffe, 1980b, and unpubl.)

Curiously, when the hemocytes of *A. astacus* and *C. maenas* were preincubated with β-1,3 glucans, the opsonizing capacity of the prophenoloxidase activating system was lost, probably because the sticky proteins had already attached to the glass surface of the coverslips and were unavailable to the bacteria.

In arthropods, phenoloxidase activity, as evidenced by melanin deposition, frequently accompanies the host cellular reactions, to wounding, parasites, foreign implants or injected test particles (Salt, 1970; Ratcliffe and Rowley, 1979; Vey, 1979), and Söderhäll (1982) has proposed that the activation process may constitute an important recognition system in these animals. It would therefore be interesting to determine whether β-1,3 glucans also have a potentiating influence on the hemocytic responses of other arthropod species, such as the silk moth, *Bombyx mori*, which possesses prophenoloxidase that can be activated by β-1,3 glucans (Ashida, 1981). Moreover, β-1,3 glucans represent just one class of non-self molecules present in the cell walls of fungi (Unestam and Söderhäll, 1977), and prophenoloxidase can be triggered by other foreign molecules, for example bacterial lipopolysaccharide (endotoxin) (Söderhäll and Häll, unpublished) which causes gelation of amoebocyte lysates of the horseshoe crab *Limulus polyphemus* (Levin and Bang, 1964).

Finally, in mammals, β-1,3 glucans are known to activate complement through the alternative pathway (Hamuro *et al.*, 1978; Reid and Porter, 1981), and Seljelid *et al.* (1981) have suggested that the increased cellular metabolism; measured by increased glucosamine incorporation, decreased nucleotidase activity and cell enlargement; induced in macrophages *in vitro* by treatment with glucans could be achieved through complement intervention. At present, direct-acting hemolytic complement has not been identified in any invertebrate group (Anderson, 1981), but Day *et al.* (1970) have predicted that some might possess the terminal components which make up the alternative pathway. In arthropods, the prophenoloxidase system, in comprising a complex enzyme cascade activated by β-1,3 glucans (Unestam and Söderhäll, 1977; Söderhäll, 1981, 1982, 1983), initiating coagulation (Söderhäll, 1981), possessing antimicrobial properties (Söderhäll and Ajaxon, 1982), and, as indicated in this study, functioning as an opsonin, shows many similarities to complement and could represent such a primitive form. Related systems may have also evolved in other invertebrates, but their description awaits further investigation.

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